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(54) Title: PROCESS FOR THE PRODUCTION OF A PROTEIN

(57) Abstract

Process for the production of a mammalian stromelysin by a recombinant DNA process. The invention also relates to specific proteins, DNA sequences, vectors, host organisms, pharmaceutical compositions, DNA probes, antibodies and to the use of stromelysin to enhance the activity of collagenase. Stromelysin may be useful therapeutically for example in the debridement of dermal ulcers, modification of scar tissue formation arising from healing of wounds and in the treatment of herniated vertebral discs.

PROCESS FOR THE PRODUCTION OF A PROTEIN

Field of the Invention

This invention relates to a process for the production of a
protein, particularly a metalloproteinase by a recombinant DNA
5   process.   The invention also relates to specific proteins, DNA
sequences, vectors, host organisms and to pharmaceutical
compositions.

Background to the Invention

The metalloproteinases are a family of enzymes produced in
10   mammalian tissues and which are believed to play an important role
in the resorption of the extracellular matrices of connective
tissues.

The family of metalloproteinases includes collagenase,
gelatinase and stromelysin which work together synergistically to
15   digest all the major macromolecules comprising extracellular
matrices.   The enzymes are found in latent proenzyme form
activatable by trypsin or 4-aminophenylmercuric acetate (APMA).
The metalloproteinases have a zinc atom at their active site and
require calcium for full activity.

20   Usually tissue metalloproteinases are coordinately synthesised,
and for many tissues a specific stimulus is needed to induce
synthesis (Reynolds J. Brit. J. Dermatology (1985) 112 715-723).
The metalloproteinases are present naturally in low concentration
and cannot be extracted in large amounts from mammalian tissues.

25   Of particular interest is stromelysin which is known to degrade
many components of the connective tissues such as proteoglycan core
protein, non-helical regions of type IV collagen, laminin,
fibronectin, elastin, gelatin and procollagens types I, II and III
and which may play a role in biological processes such as wound

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healing.   Stromelysin may be useful therapeutically in processes
such as debridement of dermal ulcers and modifying scar tissue
formation arising from the healing of wounds such as burns or
necrosis. Stromelysin may also be used in the treatment of herniated
5   vertebral discs to effect dissolution of nucleus pulposus. In order
to use stromelysin in this way it must be produced on a large scale
and at a commercially viable cost.   We provide mammalian
stromelysin in such commercially worthwhile amounts by producing it
in accordance with the invention, using recombinant DNA techniques.

10   Preprostromelysin is a large molecular weight form of
stromelysin which on secretion is processed to yield the lower
molecular weight prostromelysin.   Activation of prostromelysin by,
for example, treatment with 4-amino phenylmercuric acetate yields
the mature biologically active stromelysin.

15   Summary of the Invention

According to the present invention there is provided a process
for the production of a mammalian stromelysin comprising culturing
host cells transformed with a DNA sequence coding for the
stromelysin.

20   The term mammalian stromelysin as used herein denotes a
mammalian stromelysin having the amino acid sequence of an authentic
mammalian stromelysin, an analogue thereof, or a biologically active
peptide fragment of either of these, having the biological activity
associated with authentic mammalian stromelysin.

25   In a preferred embodiment the invention provides a process for
the production of human stromelysin comprising culturing host cells
transformed with a DNA sequence coding for human stromelysin.

The process allows the production of relatively large
quantities of stromelysin and, for the first time, facilitates
30   complete characterisation of the structure and pharmacological
properties of the protein.

The stromelysin preferably has an amino acid sequence of greater than 90 percent homology (common amino acids/total amino acids) with the sequence of amino acids 1 to 477 of human preprostromelysin as shown in Figure 4 of the accompanying drawings. More preferably, the homology is greater than 98 per cent and most preferably the human preprostromelysin has the amino acid sequence substantially as shown in Figure 4.

In addition to the complete mammalian stromelysin proteins, it is envisaged that relatively small peptide fragments of such proteins e.g. a peptide fragment of stromelysin may be useful as a metalloproteinase. Thus, in a particular embodiment of the first aspect of the invention, and where appropriate in subsequent aspects of the invention, the term stromelysin includes a peptide fragment of a stromelysin protein.

The mammalian stromelysin is suitably a methionine-stromelysin or a methionine-prostromelysin. It is currently understood that to obtain expression of a DNA sequence, the DNA sequence must possess a 5' ATG codon and the corresponding polypeptide therefore possesses an N-terminal methionine amino acid. As used herein the term "methionine-stromelysin" and "methionine-prostromelysin" denote an authentic mammalian stromelysin or prostromelysin, (or an authentic mammalian stromelysin or prostromelysin, modified or substituted to provide a functionally equivalent protein) having an N-terminal methionine residue. Preferably the methionine residue is adjacent to the N-terminal amino acid of the stromelysin or prostromelysin but may be separated therefrom by one or more amino acids provided that the protein possesses stromelysin or prostromelysin functional activity.

In a second aspect of the invention there is provided a process for the production of a mammalian stromelysin comprising producing a precursor of the stromelysin by culturing host cells transformed with a DNA sequence coding for the stromelysin precursor and cleaving the precursor to produce the stromelysin.

In a preferred embodiment of the second aspect of the invention

we pr vide a process for the production of human stromelysin
c mprising producing a precursor of human stromelysin by culturing
host cells transformed with a DNA sequence coding for the stromelysin
precursor and cleaving the precursor to produce human stromelysin.

5   The precursor protein may be a methionine-stromelysin or
methionine-prostromelysin.

           The precursor protein may be a mammalian stromelysin or a
prostromelysin with an amino terminal signal sequence. The signal
sequence may be a sequence having the effect of encouraging
10   transport of the expression products from the host cell in which the
DNA sequences have been expressed. For example, in the case of
human stromelysin, a signal sequence of amino acids such as depicted
by amino acids 1 to 17 in Figure 4 of the accompanying drawings may
be attached to the amino terminal amino acid to form
15   preprostromelysin. This signal sequence assists in the export of
the product from eukaryotic host cells and is itself cleaved from
the product during transport through the cell membrane.

           The precursor protein may be a fusion protein comprising a
heterologous protein and a mammalian stromelysin or prostromelysin
20   protein. The heterologous protein may be all or a part of a protein
capable of being produced in a host organism, preferably at a high
level. Such heterologous proteins include β-galactosidase,
chloramphenicol acetyl transferase (CAT) and the product of the trpE
gene. The fusion protein preferably includes a site susceptible to
25   selective chemical or enzymatic cleavage between the stromelysin or
prostromelysin protein and the heterologous protein. The
heterologous protein may be a yeast signal sequence and the host
organism may be a yeast. In this preferred embodiment, the yeast
host organism advantageously cleaves the fusion protein to produce a
30   mature stromelysin or prostromelysin.

           The process of the invention permits the production of
essentially pure human stromelysin, prostromelysin or prepro-
stromelysin. The term essentially pure is used to denote stromelysin
which is essentially free from other proteins of human origin.

In a third aspect the inventi n therefore provides an
essentially pure mammalian stromelysin, prostr melysin or
preprostromelysin characterised in that the mammalian stromelysin,
prostromelysin or preprostromelysin is human stromelysin,
prostromelysin or preprostromelysin.

In a preferred embodiment of the third aspect of the invention
the essentially pure human stromelysin has substantially the amino
acid sequence of from 100 to 477 as shown in Figure 4 of the
accompanying drawings, the essentially pure human prostromelysin has
the amino acid sequence of from 18 to 477 as shown in Figure 4 of
the accompanying drawings and the essentially pure human
preprostromelysin has the amino acid sequence of from 1 to 477 as
shown in Figure 4 of the accompanying drawings.

In a fourth aspect of the invention, we provide a mammalian
stromelysin or prostromelysin produced by the process of the first
aspect of the invention or a mammalian stromelysin or prostromelysin
precursor protein produced as an intermediate compound.

In a preferred embodiment of the fourth aspect of the invention
the stromelysin is human stromelysin or prostromelysin or a
precursor thereof.

In a fifth aspect of the invention, we provide a fusion protein
comprising a heterologous protein and a mammalian stromelysin or
prostromelysin.

In a preferred embodiment of the fifth aspect of the invention
the fusion protein comprises a heterologous protein and human
stromelysin or human prostromelysin.

In a sixth aspect of the invention, we provide a DNA sequence
coding for the amino acid sequence of a mammalian stromelysin or
prostromelysin , or a precursor thereof provided that when the DNA
sequence codes for rat stromelysin the DNA sequence is not the pTR1
cDNA sequence described by Matrisian et al (EMBO Journal 4
1435O· 1440 (1985)). Preferably, we provide a DNA sequence coding

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for human preprostromelysin having substantially the nucleotide
sequence from 51 to 1484 inclusive as sh wn in Figure 4 of the
accompanying drawings. Preferably we provide a DNA sequence coding
for human prostromelysin having substantially the nucleotide
sequence from 102 to 1484 inclusive as shown in Figure 4 of the
accompanying drawings. Preferably we provide a DNA sequence coding
for human stromelysin having substantially the nucleotide sequence
from 348 to 1484 inclusive as shown in Figure 4 of the accompanying
drawings.

In a seventh aspect of the invention, we provide an expression
vector including a DNA sequence coding for a mammalian stromelysin,
prostromelysin or precursor thereof. The vector is adapted for use
in a given host cell by the provision of suitable selectable markers,
promoters and other control regions as appropriate.

In a preferred embodiment of the seventh aspect of the
invention the vector includes a DNA sequence coding for human
stromelysin or prostromelysin or a precursor thereof.

In an eighth aspect of the invention, we provide host cells
transformed with a vector according to the seventh aspect of the
invention. The host cells may be any host organism which may be
transformed with a vector including a DNA sequence coding for a
mammalian stromelysin or a precursor thereof, such that expression
of the DNA sequence occurs. Suitable host cells include yeasts
(for example Saccharomyces cerevisiae) and mammalian cells in tissue
culture (for example, hamster ovary or mouse mammary tumour cells).
Preferably, where the host cell is a bacterium or a yeast, the
vector includes a DNA sequence coding for methionine-stromelysin or
methionine prostromelysin or a fusion protein including a
stromelysin or prostromelysin and when the host cell is a mammalian
cell in tissue culture, the vector preferably includes a DNA
sequence coding for a prestromelysin or a preprostromelysin.

In a ninth aspect of th invention, we provide a pharmaceutical
composition comprising a mammalian stromelysin preferably human
strom lysin and a pharmac utically acceptable

excipient. The pharmaceutical composition may be, for example, in
the form of an injectable solution or in a form suitable for topical
administration. The pharmaceutical composition may contain a
stromelysin or a prostromelysin preferably human stromelysin or
human prostromelysin which may be activated _in vitro_ or _in vivo_ to
give biologically active stromelysin.

In a preferred embodiment of the ninth aspect of the invention
we provide a pharmaceutical composition containing an effective
amount of a mammalian stromelysin or prostromelysin , preferably
human stromelysin or prostromelysin, for use in the debridement of
dermal ulcers, modification of scar tissue formation arising from
the healing of wounds such as burns and necrosis and in the
treatment of herniated vertebral discs.

In a tenth aspect of the invention we provide a process for the
production of a pharmaceutical composition comprising bringing a
mammalian stromelysin or prostromelysin, preferably human
stromelysin or prostromelysin into association with a
pharmaceutically acceptable carrier.

In an eleventh aspect the invention provides a method of
therapy comprising treating a patient with an effective amount of a
mammalian stromelysin preferably human stromelysin, for instance for
debridement of dermal ulcers, modification of scar tissue formation
arising from the healing of wounds such as burns and necrosis or
treatment of herniated vertebral discs.

Preferably, the stromelysin produced in accordance with the
invention will have a useful pharmacological effect without
significant antigenic reaction with the immune system. In
particular the compounds may be used in the debridement of dermal
ulcers, modification of scar tissue formation arising from the
healing of wounds such as burns and necrosis and in the treatment of
herniated vertebral discs.

The DNA sequence sequence coding for human stromelysin as
described in Figure 4 may be used to design DNA probes for use in

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identifying the over expression of stromelysin indicative f
connective tissue disease states and the invention extends to such
DNA probes.

In a twelvth aspect the invention provides a DNA hybridisation
probe comprising a sequence of nucleotides selected from the
nucleotide sequence from 1 to 1810 as shown in Figure 4 of the
accompanying drawings.

In a further aspect the invention provides an antibody having
specificity for an antigenic determinator of a mammalian
stromelysin.   The antibody may be a polyclonal or monoclonal
antibody.   The antibody may be labelled with a detectable label.

Stromelysin is shown to be able to enhance the activity of
collagenase after its activation with, for example, trypsin or APMA
and the invention extends to the use of stromelysin for this purpose.

Brief Description of the Drawings

The invention is further described by the following non-
limiting examples which refer to the accompanying diagrams Figures 1
to 9 and Tables 1 and 2, short particulars of which are given below.

Figure 1   shows the N. terminal amino acid sequence of
             rabbit APMA activated stromelysin (a) and the
             oligo-nucleotide probe based on this sequence.
             The 50 base oligonucleotide probe was made in
             two 25 base halves (b and c) and the
             complementary 26-mer (d) was synthesised in
             order to facilitate their ligation.   The
             numbering of the amino acids refers to their
             position in the cDNA predicted rabbit
             stromelysin sequence (see Fig 2)

Figure 2   Shows the partial nucleotide sequence of rabbit
             stromelysin and the predicted amino acid sequence

Figure 3   Shows the restriction maps of 3 human stromelysin
              cDNAs

Figure 4   Shows the restriction map and nucleotide sequence
              of human stromelysin cDNA and the predicted
5   amino acid sequence

Figure 5   Shows electrophoretic analysis of the products
              of activation of stromelysin purified from:
              panel A - human gingival fibroblast culture
                        medium.
10   panel B - that secreted by C127 cells transfected
                        with stromelysin-containing vector
              panel C - that secreted by COS cells transfected
                        with stromelysin-containing vector

Figure 6   Shows a graph of the time course of activation
15   of C127 cell secreted prostromelysin by various
              concentrations of trypsin or APMA

Figure 7   Shows electrophoretic analysis of the products
              of C127 prostromelysin activation with APMA

Figure 8   Shows a graph of the effect of different amounts
20   of purified human fibroblast prostromelysin or
              prostromelysin secreted by C127 cells on the
              activation by trypsin of:

              A). procollagenase secreted by transfected C127
              cells, and

25   B). procollagenase purified from human gingival
              fibroblast culture

Figure 9   Shows electrophoretic analysis of procollagenase
              from human gingival fibroblast culture medium
              activated by APMA, APMA + stromelysin, trypsin,
30   or trypsin + stromelysin.

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Table 1   Amino acid composition of human pr stromelysin

Table 2   Amino acid composition of human stromelysin

Detailed Description of the Embodiments

In the following example, prostromelysin is activated by
treatment with 4-amino phenylmercuric acetate to yield mature
stromelysin. It will be appreciated, however, that by using
alternative methods of cleavage of the proenzyme such as enzymic
digestion, different forms of active stromelysin may be produced and
these are included within the scope of the present invention.

1.1 THE ISOLATION OF A RABBIT cDNA ENCODING STROMELYSIN

4-aminophenylmercuric acetate (APMA) activated (Cawston and
Murphy in: Methods in Enzymology, Volume 80 pp711-722 (1981))
rabbit fibroblast stromelysin was purified from the culture media of
calvariae (Galloway, W. A. et al Biochem. J. 209, 741-752 (1983)).
It was reduced and carboxymethylated and subjected to N-terminal
sequencing by automated Edman degradation on an Applied Biosystems
gas-phase sequenator. The 50 base oligonucleotide probe was
designed according to previously described rules to be capable
theoretically of hybridizing to both rabbit stromelysin and
collagenase cDNAs (Grantham R. et al Nucleic Acis Res. 9, r43-r47
(1981) and Lathe, R. J. Molec. Biol. 183, 12252-12258 (1983)). It
was synthesized as two continuous 25 mers (b and c, Figure 1) by
automated solid-phase phosphotriester chemistry followed by
purification by HPLC (Patel T. P. et al Nucleic Acids Res. 10,
5605-5619 (1982)). A complementary 26 mer (d) was also made (see
Fig. 1).

Rabbit fibroblasts from explants of normal synovium were grown
to confluence in Dulbecco's modified Eagle's medium (DMEM)
supplemented with 10% fetal calf serum (FCS). After washing they
were then maintained in serum-free media for 48 h in the presence of

50-200 ng ml^{-1} 12 - $\underline{O}$ - tetradecan ylphorbol - 13 - acetate (TPA).
mRNA was isolated fr m approximately 5.10^7 cells by the guanidinium
isothiocyanate/hot phenol method (Maniatis $\underline{et\ al}$ 1982 in. Molecular
Cloning: A laboratory Manual; Cold Spring Harbor laboratory, New
York), followed by oligo (dT)-cellulose column chromatography (Aviv
and Leder, 1972 PNAS USA $\underline{69}$ 1408-1412). Standard procedures were
used to synthesise cDNA (Gubler and Hoffman, 1983 Gene $\underline{25}$ 263-269)
and a library of 75,000 plaques was established by using EcoRl
linkers to ligate the cDNA into λ gt10 essentially as described by
Huynh $\underline{et\ al}$ in DNA Cloning Vol 1 ed. D. M. Glover, IRL Press p49-78.

The oligonucleotide probe was prepared by first subjecting to a
kinase reaction the 25 mer labelled (c) fig 1 and then ligating it
to the 25-mer labelled (b) in the presence of the complementary
26-mer (d). The 50-base oligonucleotide probe resulting from the
ligation was purified from a denaturing polyacrylamide gel (Maxam A.
M & Gilbert W. in Methods in Enzymology, Volume 65, pp 499-560
(1980)) and then kinased to a specific activity of approximately
3.10^8 Ce cpm µg-^{1}.

The plaques were transferred to nitrocellulose filters,
denatured, and then prehybridised in 5 x SSC, 5 x Denhardt's
solution, 50mM NaH2PO4, 100 µgml denatured salmon sperm DNA for 6h
at 60°C. Hybridisation was then undertaken for 16h at 40°C in an
identical buffer to which had been added 0.1% SDS and the probe DNA
at approximately 4ng ml^{-1}. The filters were then washed in 6 X SSC
before a final stringent wash (1 X SSC, 0.1% SDS for 15 min at 50°C)
followed by autoradiography for 16 h with an intensifying screen.
10 putatively positive plaques were identified of which one gave
positive signals after a second screening undertaken as described
above. DNA was isolated and the cDNA insert was subcloned into
plasmid pSP64 (Melton D. A. $\underline{et\ al}$ Nucleic Acids Res. $\underline{12}$, 7035-7056
(1984)). The complete nucleotide sequence of the cDNA was
determined by the dideoxy method (Sanger F., Nicklen S. & Coulson A.
R. Proc. Natl. Acad. Sci USA $\underline{74}$ 5463-5467 (1977)) after subcloning
restriction fragments into M13 and transformation into JM101
(Messing J. & Vieira, J. Gene $\underline{19}$ 269-276 (1982)).

As shown in fig 2 the cDNA is 532 nucleotides in length and
although lacking a poly A tract do s have an open reading frame
encoding 170 amino acids. Residues 101-127 are identical with the
N-terminal amino acid sequence of rabbit stromelysin (Fig 1a) and
5   the amino acid sequence encoded by the cDNA therefore represents the
N-terminal third of rabbit stromelysin. This cDNA was therefore
used as a probe to identify the human stromelysin cDNA. It was
labelled with ^{32}P α dATP and Klenow fragment using the random
hexanucleotide method (Feinberg and Vogelstein, 1983, Anal. Biochem,
10       $\underline{32}$ 6-13) to a specific activity of 2.10^7 Ce cpm µg^{-1}.

1.2 <u>ISOLATION OF A HUMAN cDNA ENCODING STROMELYSIN</u>

Human fibroblasts derived from gingival explants (passage 3 to
6) were cultivated in DMEM supplemented with 0.2% lactalbumin
hydrolysate and 5% partially purified pig IL-1. After 48h mRNA was
15   isolated from approximately 5×10^8 cells by the guanidinium .
isothiocyanate/CsCl method (Maniatis <u>et al</u> 1982 in Molecular
Cloning: A laboratory Manual; Cold Spring Harbor Laboratory, New
York). The mRNA was used to generate a library of 200,000
recombinant plaques in λgt10 essentially as described above.   The
20   plaques were transferred to nitrocellulose, denatured, and then
prehybridised in 6 X SSC, 50% formamide, 5 X Denhardt's solution,
100 µg ml^{-1} denatured salmon sperm DNA for 16 h at 42°C.   They
were then hybridised for 48h at 42°C in an identical buffer to which
had been added 0.1% SDS and the rabbit stromelysin probe cDNA at a
25   final concentration of approximately 17 ng ml^{-1}.   The filters were
then washed with the highest stringency being 2 X SSC, 0.1% SDS at
42°C for 30 minutes before autoradiography with an intensifying
screen for 48h.   21 putatively positive plaques were identified of
which 16 gave a clear positive signal after a second screening
30   undertaken as described above.   The cDNA insert of representative
plaques was isolated and subcloned into plasmid pSP64 (Melton D. A.
<u>et al</u> Nucleic Acids Res. <u>12</u>, 7035-7056 (1984)). Restriction mapping
of the largest inserts confirmed that they were related overlapping
DNA segments (Fig. 3) and their nucleotide sequence was compiled by
35   the dideoxy method (Sanger F., Nicklen S. &

Coulson A. R. Proc. Natl. Acad. Sci USA <u>74</u> 5463-5467 (1977)) after

subcloning restriction fragments into M13 and transformation into

JM101 (Messing J. & Vieira, J. Gene <u>19</u>, 269-276 (1982)). This

information is shown in Fig 4, where the solid arrows indicate

5   direction of dideoxy sequencing and the broken arrows show

directions of sequencing by the Maxam and Gilbert method (Maxam,

A.M. and Gilbert, W., Methods Enzymol. (1980) <u>65</u> 499-560).

1.3 <u>IDENTIFICATION OF RAT cDNA ENCODING STROMELYSIN</u>

We have discovered a high degree of homology between rabbit

10   stromelysin and another amino acid sequence predicted from a rat

cDNA, referred to as pTR1 (Matrisian L. M. <u>et al</u> EMBO Journal <u>4</u>,

1435-1440 (1985)). This cDNA corresponds to a mRNA which is highly

enriched in normal fibroblasts after infection with either polyoma

virus or rous sarcoma virus or transfection with either the middle t

15   oncogene or the cellular oncogene H-ras. The same mRNA was also

reported to be specifically induced after exposure of fibroblasts to

EGF (Matrisian L. M. <u>et al</u> EMBO Journal <u>4</u>, 1435-1440 (1985)). The

predicted Mr of the rat protein encoded by pTR1 is approximately

53000, in close agreement with the <u>in vitro</u> translation product of

20   rabbit stromelysin mRNA (Frisch S. M., Chin J. R. & Werb Z. J. Cell

Biol. <u>97</u>, (2, Pt. 5): 430a (Abstr.) (1983)), and data not shown].

We conclude therefore, that the protein encoded by pTR1 is rat

stromelysin. The identification of pTR1 as rat stromelysin allows,

for the first time, the production of rat stromelysin using

25   recombinant DNA techniques.

2. <u>NUCLEOTIDE SEQUENCE OF HUMAN STROMELYSIN cDNA AND THE</u>
   <u>PREDICTED AMINO ACID SEQUENCE</u>

The cDNA is 1825 nucleotides in length and appears to be

complete at the 3' end because it has a poly A tail preceded by the

30   polyadenylation signal AATAAA (Proudfoot and Brownlee, 1981, Nature,

<u>252</u> 359-362). It contains an open reading frame (nucleotides 51 to

1481) which translates into a polypeptide of 477 amino acids.

Evidence that the cDNA encodes stromelysin comes from the finding

that 24 of the 27 amino acids encoded by nucleotides 348 to 428 are

identical with and in the same position as the 27 amino acids
identified by N-terminal sequencing the APMA activated rabbit
stromelysin (Fig 1a and Fig 4). Furthermore most of the amino acids
in the N-terminal third of the molecule are identical with and in
the same position as those amino acids predicted from the rabbit
partial stromelysin cDNA.

Hydrophobicity plots suggest that human stromelysin is rather
water soluble except for a hydrophobic N-terminal sequence of 17
amino acids. This is consistent with these amino acids being the
hydrophobic core of a signal sequence which is cleaved during
secretion to liberate mature protein. Since it is thought that the
metalloproteinases are secreted as proenzymes (Harris E. D. et al
Collagen Rel Res 4 493-512 (1984)) we conclude that the amino acids
found between the signal sequence and the phenylalanine (F) at the
N-termini of the APMA - activated form of rabbit stromelysin
(residue 100 in the human stromelysin sequence) are cleaved off
during activation. Preprostromelysin is therefore considered to be
comprised of amino acids 1-477, prostromelysin is considered to be
comprised of amino acids 18-477 and stromelysin is considered to be
comprised of amino acids 100-477. The amino acid composition of
prostromelysin and stromelysin together with the molecular weight of
the core proteins is shown in tables 1 and 2 respectively.
Potential glycosylation sites within the sequences exist, and it is
envisaged that expression of these proteins in appropriate cells
(see below) may give rise to glycosylated products.

3. THE PRODUCTION OF STROMELYSIN

3.1 EXPRESSION IN E. COLI

The expression of stromelysin or prostromelysin with an additional
NH_2-terminal methionine residue (met-stromelysin or met-
prostromelysin) in E. coli can be achieved by using oligonucleotide
linkers to join the mature stromelysin or prostromelysin encoding
sequence described in Section 2 above and shown in Figure 4 with a
promoter, Shine Dalgarno sequence and an initiating ATG codon. The
E. coli trpE promoter and Shine Dalgarno sequence may be used.

TABLE 1

Amino Acid Composition of Human Prostromelysin

Residue	Number	%	Weight	%
F	32	6.96	4704	9.02
L	38	8.26	4294	8.23
I	20	4.35	2260	4.33
M	6	1.30	786	1.51
V	28	6.09	2772	5.31
S	27	5.87	2349	4.50
P	36	7.83	3492	6.69
T	27	5.87	2727	5.23
A	27	5.87	1917	3.68
Y	17	3.70	2771	5.31
H	13	2.83	1781	3.41
Q	9	1.96	1152	2.21
N	16	3.48	1824	3.50
K	32	6.96	4096	7.85
D	36	7.83	4140	7.94
E	30	6.52	3870	7.42
C	3	0.65	309	0.59
W	8	1.74	1488	2.85
R	23	5.00	3588	6.88
G	32	6.96	1824	3.50
TOTAL	460		52162	

The numbers 5, 10, 15, 20 appear in the left margin alongside the rows.

TABLE 2

Amino Acid Composition of Human Stromelysin

Residue	Number	%	Weight	%
F	30	7.94	4410	10.31
L	30	7.94	3390	7.92
I	19	5.03	2147	5.02
M	3	0.79	393	0.92
V	19	5.03	1881	4.40
S	24	6.35	2088	4.88
P	32	8.47	3104	7.25
T	24	6.35	2424	5.67
A	25	6.61	1775	4.15
Y	13	3.44	2119	4.95
H	12	3.17	1644	3.84
Q	6	1.59	768	1.79
N	14	3.70	1596	3.73
K	22	5.82	2816	6.58
D	28	7.41	3220	7.53
E	25	6.61	3225	7.54
C	2	0.53	206	0.48
W	8	2.12	1488	3.48
R	17	4.50	2652	6.20
G	25	6.61	1425	3.33
TOTAL	378		42789	

These sequences are present f r example on plasmid pCT54 into
which DNA sequences encoding proteins of interest can be inserted in
a ClaI site (Emtage et al (1983), Proc. Nat. Acad. Sci. USA, 80,
3671-3675). However, other plasmids also harbouring these or other
5   promoter and Shine Dalgarno sequences but which are amplifiable and
from which expression can be tightly regulated may also be used
(see, for example, European patent application EP-A2-0121386). The
stromelysin or prostromelysin coding sequence without the signal
coding sequence but with an additional N-terminal methionine codon,
10   may be inserted into the E. coli expression vector pMG196. This may
be achieved by standard recombinant DNA techniques (Maniatis et al
(1982), In: Molecular Cloning: A Laboratory Manual, 390-433).
Expression of stromelysin or prostromelysin may also be achieved
using analogous techniques to those described for expression of TIMP
15   in European patent application No. 86300042.8.

3.2 PURIFICATION OF STROMELYSIN FROM E. COLI CELLS

E. coli cells harbouring a plasmid constructed as described
above, when grown under optimal conditions for expression, will
produce met-stromelysin or met-prostromelysin at levels of up to 10%
20   of total cellular protein.

The soluble protein fraction from crude E. coli extracts is
assayed for stromelysin activity essentially as described by Galaway
et al (1983) Biochem. J. 209 741-752. A part or whole of the
met-stromelysin expressed in E. coli may be in an insoluble form and
25   therefore not detected in the above assay. In such circumstances,
it is solubilised and activated prior to assay and purification.
One example of how this can be achieved in relation to
methionine-prochymosin production is described in our co-pending
International patent application PCT/GB 83/00152 (published as WO
30   83/04418) and in published British patent application GB 2100737A.
Having obtained soluble, active, partially purified stromelysin it
is further purified using standard pr tein purification techniques,
as described for example by Galaway et al (1983) Biochem. J. 209
741-752.

3.3 EXPRESSION IN YEAST

Using standard recombinant DNA techniques in a similar manner
to those used to achieve expression in E. coli, plasmid vectors
suitable for expression of stromelysin in yeast are constructed.

5   The constructions are based, for example, on the vectors described
in co-pending published European patent application EP-A2-0073635.
They contain the stromelysin or prostromelysin encoding sequence
flanked by the yeast phosphoglycerate kinase (PGK) promoter and the
PGK gene 3' untranslated end. The orientation of the stromelysin or

10   prostromelysin cDNA with respect to the PGK promoter is such that it
ensures expression of mature stromelysin or prostromelysin with an
additional NH_2-terminal methionine residue as described for
expression in E. coli. Alternatively, pre-stromelysin or
prostromelysin may be expressed by joining the stromelysin or

15   prostromelysin encoding sequence to the vector such that the
stromelysin signal sequence is left in place. Expression of a
fusion protein between a yeast signal sequence (for example, the
yeast α-Factor signal sequence (Kurjan and Herskowitz, (1982),
Cell, 30, 933-943), and mature stromelysin or prostromelysin can be

20   achieved through the use of appropriate linkers. These plasmid DNAs
are introduced into yeast cells, for example, by the method of Beggs
(Nature, (1978), 275, 104-109).

3.4 PURIFICATION OF STROMELYSIN FROM YEAST CELLS

Yeast cells containing these plasmids, when grown under optimal

25   conditions for stromelysin expression, will produce up to 5% of
total cellular protein as stromelysin or prostromelysin. Depending
on the alternatives described above, stromelysin or prostromelysin
may or may not be secreted from the yeast cells. Expressed
stromelysin is quantified, assayed and purified essentially as

30   described above if produced intercellularly. If secreted it is
purified from cell supernatants by standard protein purification
techniques (Galaway et al (1983) Biochem. J. 209 741-752).

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3.5 EXPRESSION OF STROMELYSIN IN CULTURED ANIMAL CELLS

     The prepr str melysin encoding cDNA was inserted into the
different kinds of expression vector designed specifically for: 1.
transient expression in COS cells (Gluzman, Y. (1981) Cell 23 175-
5   182) and 2. Stable expression in C127 cells (Lowy, D.R. et al J.
Virol. (1978) 26 291-298).   The necessary DNA manipulations
employed standard techniques (Maniatis et al In. Molecular Cloning,
A Laboratory Manual, 1982, Cold Spring Harbor Laboratory, New
York).   The cDNA was tailored for insertion into the vectors by
10   first converting the Ava I site encoded by nucleotides 1591-1596
(Figure 4) into an EcoR1 site.   This was achieved by cleaving the
cDNA with Ava I, filling in with T4 polymerase followed by addition
of an EcoR1 linker.   On cleavage with EcoR1 a 1600 bp EcoR1
fragment encoding preprostromelysin extending from the 5' EcoR1 site
15   encoded by nucleotides 1-6 in Figure 4 to the newly created EcoR1
site is generated.   This fragment was purified from a 1% low
gelling temperature agarose gel and then ligated between the SV40
late promoter and the SV40 early polyadenylation regulatory elements
in a "poison minus" derivative of a pBR322 COS cell vector (Lusky, M
20   and Botchan, M.R., Nature, 1981, 293 79-81).   Vector DNA containing
the preprostromelysin sequence in the appropriate orientation with
respect to the SV40 late promoter was purified from cesium chloride
gradients and transfected into COS cells using DEAE dextran (Lopata,
M.A. et al Nucleic Acids Res., 1984, 12 5707-5717).   Serum-free
25   supernatants were harvested 72 hours after transfection.

     For expression in C127 cells the preprostromelysin sequence
with the early SV40 polyadenylation regulatory elements was removed
on a PstI to BamH1 fragment from the COS cell vector and put under
the control of the mouse metallothionine I promoter in a bovine
30   papilloma virus (BPV) based vector.   This arrangement was
essentially as described for the expression of tissue inhibitor of
metalloproteinase (TIMP), Docherty et al Nature 1985, 318 66-69 and
UK Patent Application GB 2169295A.   The BPV vect r encoding
preprostromelysin was introduced into C127 cells by the calcium
35   phosphate coprecipitation method (Wigler, M. et al 1978, Cell 14
725-731) and CdCl₂ (20µM) - and ZnCl₂ (20µM) - resistant

foci were selected. Stromelysin producing cell lines were
identified after 21 days and used to generate s rum-free culture
medium.

4. STROMELYSIN ACTIVATION, ASSAY AND ELECTROPHORETIC ANALYSIS

5   The stromelysin produced as described above was activated with
either trypsin or APMA. Trypsin was incubated with the stromelysin
at ratios such as 1:100 to 1:1 respectively followed by the addition
of a ten-fold excess of soya-bean trypsin inhibitor. Concentrations
of trypsin such as 0.1 to 10μg ml^{-1} were employed and the
10   temperatures and time of incubation were as described below.
Treatment with 4-aminophenylmercuric acetate (APMA) was at 1 to 2 mM
for various times as indicated below, at 37°C. Stromelysin
activity was assayed using ^{14}C-acetylated casein (Galloway, W.A.,
et al Biochem. J. 1983, 209 741-752) at 35°C for 1 or 4 h.   1 unit
15   of stromelysin degrades 1μg of casein min^{-1} at 37°C.

          Stromelysin before and after activation as described above and
in more detail below was treated with 20mM EDTA, reduced with 500mM
2-mercaptoethanol and run on 10% polyacrylamide mini gels in the
presence of SDS. Gels were electro-transferred to nitrocellulose
20   and the enzyme bands visualised using an antisera against rabbit
stromelysin which was able to detect human stromelysin (Murphy, G.
et al Collagen Rel. Res. 1986 6 351-364) and a peroxidase-labelled
second antibody (Hembry, R.M., et al J. Cell Sci. 1985 73 105-119).

          As shown in Figure 5, prostromelysin preparations A, purified
25   from human gingival fibroblast culture medium, B, secreted by C127
cells transfected with the stromelysin-containing vector and C,
secreted by COS cells transfected with the stromelysin-containing
vector, were treated as follows: 1. no treatment; 2. incubation
with 1mM APMA for 2h at 37°C; 3. incubation with 10/μg/ml trypsin
30   for 30 min at 37°C; 4. as 3. with addition of soya bean trypsin
inhibitor after 30 min and further incubation for 2h at 37°C; 5.
incubation with 100μg/ml trypsin for 10 min at 4°C; 6. incubation
with 10μg/ml trypsin for 5 min at 4°C;

- 21 -

7. as 6. with addition of soya bean trypsin inhibitor after 5 min
and further incubation for 2h at 37°C.   The samples were
electrophoresed n a 10% polyacrylamide gel in SDS and reducing
conditions, electro-blotted onto nitrocellulose and visualised using
5   a sheep anti rabbit stromelysin antibody, and a peroxidase labelled
rabbit anti sheep IgG.

As shown in Figure 6 prostromelysin secreted by C127 cells
transfected with the stromelysin-containing vector was incubated
with A, (●) 0.1µg/ml trypsin, (Δ) 1µg/ml trypsin, B, (□) 10µg/ml
10   trypsin, C, (∇) 100µg/ml trypsin at 37°C (closed symbols) or 4°C
(open symbols) in a volume of 20µl, or D, (◆) with 2mM APMA at
37°C.   Activity elicited was assayed by degradation of ^{14}C-casein
in a 1h assay at 37°C, after addition of soya bean trypsin
inhibitor.   Stromelysin incubated without trypsin or APMA had no
15   activity.

Prostromelysin was activated with APMA (as described in Figure
6) for varying lengths of time.   The samples were then treated with
20mM EDTA and electrophoresed under reducing conditions on a 10%
polyacrylamide gel containing SDS and electroblotted and visualised
20   as described in Fig. 5 and Methods.   Figure 7 shows the SDS
analysis where Track 1. 0 min;   Track 2. 30 min;   Track 3. 1h;
Track 4. 2h;   Track 5. 4h;   Track 6. 15h.

The pro-forms of either purified natural stromelysin from human
gingival fibroblasts, or the recombinant enzyme secreted by COS or
25   C127 cells had an identical Mr of 57000, with a minor 60000 component
(probably due to glycosylation (Nagase, H. et al (1983) Biochem. J.
214 281-288);   Figure 5, lanes A1, B1, C1).   These forms were
completely inactive against casein or other substrates (Galloway, W.A.
et al (1983) Biochem. J. 209 741-752) but could be activated by
30   trypsin, (e.g. C127 stromelysin, Fig. 6A-C) or less efficiently by
APMA, to degrade these substrates (Figure 6D).   Trypsin activation
was optimal over a wide range of concentrations;   activation could
be effected at 4°C, with marginally slower kinetics (Figure 6A-C).
Only very high levels of trypsin inactivated stromelysin.
35   Activation by APMA was slower, achieving 58% (COS) to 90% (C127 and

natural) of the trypsin value.   Although maximum APMA activation
was attained in 4-8 h at 37°C (Figure 6), it was found that
activation was dependent upon stromelysin concentration (73% of
optimal at a ten fold dilution and 33% at a one hundred fold
5   dilution of a 50 unit/ml preparation).   Hence, very low
concentrations of enzyme, such as the COS cell culture media were
probably not maximally activated even after 15 h incubation.

Analysis of the effect of these treatments on the
prostromelysin by gel electrophoresis and immuno blotting showed
10   that a reduction in Mr occurred to yield two major species of Mr.
50000 and 48000 slowly in the case of APMA (e.g. C127 stromelysin,
Figure 7) and very rapidly in the case of trypsin (Figure 5 A3;
B3,5; C3,5).   At longer incubation times with APMA traces of an
Mr. 28000 form of stromelysin were generated (Figure 7).   Plasmin
15   could also be used to generate bands of the same Mr as those
produced by trypsin (data not shown).   It was noted that optimal
activation (as documented in Figure 6) did not require complete
conversion of the upper doublet to the lower doublet (Figure 5;
lanes A6, A7; Figure 3, lane 4).   Using collagenase, Stricklin
20   et al (Biochemistry 22 63-68 (1983)) have reported similar
observations with activity detectable prior to an Mr loss.   It has
been proposed that conformational changes in the intact molecule
occur, leading to subsequent self-cleavage.   The activity of
stromelysin elicited was 360 units/mg.

25   5. COLLAGENASE ACTIVATION ELECTROPHORETIC ANALYSIS AND ASSAY

The collagenase was activated as described for stromelysin or
as detailed below.   Electrophoretic analysis was undertaken as
described for stromelysin except that enzyme bands were visualised
using anti-human collagenase antisera followed by a peroxidase-
30   labelled second antibody (Hembry, R.M. et al J. Cell Sci. 1985, 73
105-119).   The collagenase was assayed by the ^{14}C-acetylated
collagen diffuse fibril assay at 35°C for 4 hours (Whitham, S.E. et
al Biochem. J. 1986, 240 913-916).   1 unit of collagenase degrades
1μg of type I collagen min^{-1} at 35°C.

Figure 8 sh ws the results when procollagenase preparations, A,
s creted by C127 cells transfected with the collagenase containing
vect r and B, purified from human gingival fibroblast culture m dium
were incubated with 10µg/ml trypsin at 37°C for 30 min in the
5   presence of varying amounts of (●) purified human fibroblast
prostromelysin or (■) prostromelysin secreted by C127 cells
transfected with the stromelysin-containing vector, followed by the
addition of excess soya bean trypsin inhibitor.   Open symbols
denote the effect of the corresponding activated stromelysin in the
10   absence of trypsin.   Activity was. assayed using ^{14}C-type I
collagen at 35°C for 4h.   Results are expressed as the fold
increase elicited by stromelysin relative to the activity with
trypsin alone.

Figure 9 shows electrophoretic analysis of procollagenase
15   purified from human gingival fibroblast culture medium treated as
follows: 1. no treatment; 2. incubation with 1mM APMA at 37°C for
2h; 3. as 2, in the presence of purified stromelysin; 4.
incubation with 10µg/ml^{-1} trypsin at 37°C for 30 min, followed by
addition of soya bean trypsin inhibitor; 5. as 4. in the presence
20   of stromelysin.

Purified natural procollagenase has an Mr of 55000 with a minor
component of Mr 59000 upon analysis by SDS polyacrylamide gel
electrophoresis and blotting with a specific antiserum to
collagenase (Figure 9; lane 1).   The procollagenase could be
25   activated by either APMA or trypsin treatment behaving precisely as
described by Stricklin et al (Biochemistry 22 63-68 (1983)) with a
fall in Mr of 10,000 (Figure 9, lanes 2 and 4).   The activity
elicited was very low, in the region of 320 units/mg.   A similar
observation was made by Vater et al (J. Biol. Chem. 258 9374-9382
30   (1983)) for rabbit procollagenase.   However, the inclusion of either
purified or recombinant human prostromelysin in the activation
mixture enhanced the collagenase activity by up to 8 fold (Figure
8B).   Similar effects f stromelysin on the molecular weight and
activity of recombinant C127 cell derived human collagenase were
35   also obtained.   With the recombinant collagenase the stromelysin
brought about a 12 fold enhancement in activity (Figure 8A).

Addition of previously activated stromelysin to th collagenase
after trypsin treatment had less effect on the final collagenase
activity detectable (data not shown). Active stromelysin alone
elicited similar collagenase activities to those with trypsin
5   alone. The ratio of stromelysin:collagenase for efficient
activation was in the order of an excess of 2moles or more per
mole. It is possible that higher specific activity stromelysin
would be a more efficient activator.

Analysis of the changes occurring during these treatments
10   showed that the procollagenase, underwent stepwise reductions in Mr
APMA and trypsin generated two bands of about 50000 and 45000
(Figure 9, lanes 2 and 4) which were converted to bands of 48000 and
43000 in the presence of stromelysin (Figure 9; lanes 3 and 5).
The same band patterns were generated by both natural and
15   recombinant stromelysin. Activated stromelysin alone appeared to
produce very small changes in procollagenase of Mr about 500, with
limited further conversion to 48000 and 43000.

CLAIMS

1.   A process for the production of a mammalian stromelysin
comprising culturing host cells transformed with a DNA sequence
coding for the stromelysin.

5   2.   A process for the production of a mammalian stromelysin
comprising producing a precursor of the stromelysin by culturing
host cells transformed with a DNA sequence coding for the stromelysin
precursor and cleaving the precursor to produce the stromelysin.

3.   An essentially pure mammalian stromelysin, prostromelysin or
10   preprostromelysin characterised in that the mammalian stromelysin,
prostromelysin or preprostromelysin is human stromelysin,
prostromelysin or preprostromelysin.

4   A mammalian stromelysin or prostromelysin produced by a process
according to Claim 1.

15   5.   A fusion protein comprising a heterologous protein and a
mammalian stromelysin or prostromelysin.

6.   A DNA sequence coding for the amino acid sequence of a
mammalian stromelysin or prostromelysin or a precursor thereof
provided that when the DNA sequence codes for rat stromelysin the
20   DNA sequence is not the pTR1 cDNA sequence.

7.   An expression vector including a DNA sequence coding for a
mammalian stromelysin, prostromelysin or precursor thereof.

8.   Host cells transformed with a vector according to Claim 7.

9.   A pharmaceutical composition comprising a mammalian stromelysin
25   and a pharmaceutically acceptable excipient.

10. A method of therapy comprising treating a patient with an
effective amount of a mammalian stromelysin.

Fig. 1

a.
```
  F   S   T   F   P   G   T   P   K   W   T   K   T   H   L   T   Y   R   I   V   N   Y   T   P   D   L   P
 101                                                                                                   127
```

b.
5'-TGGAACCAGACCCACCTGACCTACAGGATTGTGAACTACACCCCCGACCT-3'

c.

d. GTGGACTGGGATGTCCTAACACTTGAT

Fig. 2

```
        H   K   K   L   F   T   L   L   L   C   V   A   L   C   S   A   Y   F   L   D   G   A   S   R   D   A   V   T   N   N   D
CGAGCTGAGTAAAGGCCAATGGAAATGAAAACTGTTCCAACCCTGCTACTGTGCTGTGGCGGCTTGCTGTCAGCCTATCCACTGGATGGGAGCCTCAAGGGATGCAGACACCACCAACAATGG
        10          20          30          40          50          60          70          80          90          100         110         120

        L   L   Q   Q   Y   L   E   N   Y   Y   N   L   E   K   D   V   K   Q   F   V   K   K   R   N   S   S   P   V   V   K   I   Q   E   H   Q   K   F   L   G
ACCTTCTTCAGCAATATCTGGAAAACTACAACCTTGAAAAAGATGTGAAACAGTTTGTTAAAAGAAAGGACAGTAGTCCTGTTGTGAAAATCCAAGAAATGCAGAAGTTCCTTG
        130         140         150         160         170         180         190         200         210         220         230         240

        L   E   V   T   G   K   L   S   N   T   L   E   V   I   R   K   F   R   C   G   V   P   G   V   G   H   F   S   F   P   G   T   P   K   W   T   K   T
GCTGGAGGTGACAGGCCAAGCTGGACTCCAACACCCTGGAGGTGATACGGAAGTTCCGATGTGGCGTTCCTGATGGTGGTCACTTCAGTAC-TTCCCTGGCACCCCAAGTGGACAAAA
        250         260         270         280         290         300         310         320         330         340         350         360

        H   L   T   Y   R   I   V   N   Y   T   P   D   L   P   R   D   A   V   D   A   A   I   E   K   A   L   K   V   E   E   V   T   P   L   T   F   S   R   K
CTCACCTACTTACAGGATTGTGAATTACACACCGGATCTGCCAAGAGATGCCGTTGATGCTGCCATTGAGAAAGCTCTGAAAGTCTGAAAGTCTGGGAGGAGGTGACTCCACTCACATTCTCCAGGA
        370         380         390         400         410         420         430         440         450         460         470         480

        Y   E   G   E   A   D   L   H   I   S   F   G   V   R   G   N   S
AGTACGAAGGAGAGGCTGACATAATGATCTCTTTTGGAGTCCGAGGGAATTC
        490         500         510         520         530
```

Fig. 3
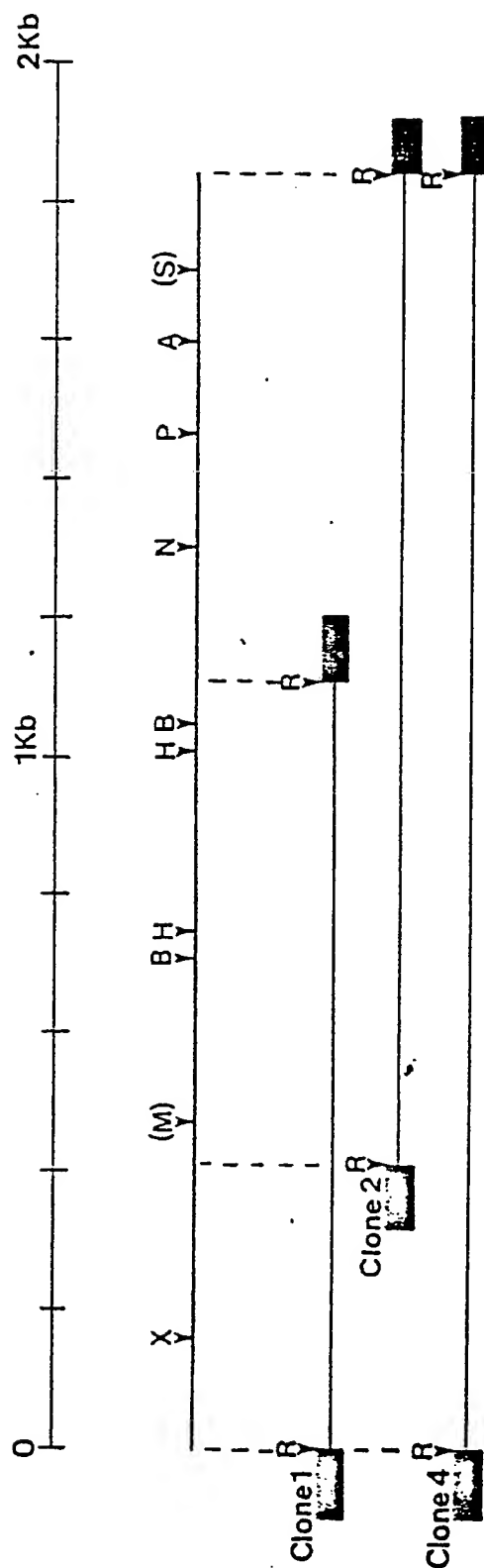
( ) = other sites for these enzymes
      are on map but not shown

X = XbaI   N = NcoI
M = MboII   P = PvuII
B = BalI   A = AvaI
H = HindIII   S = Sau3A
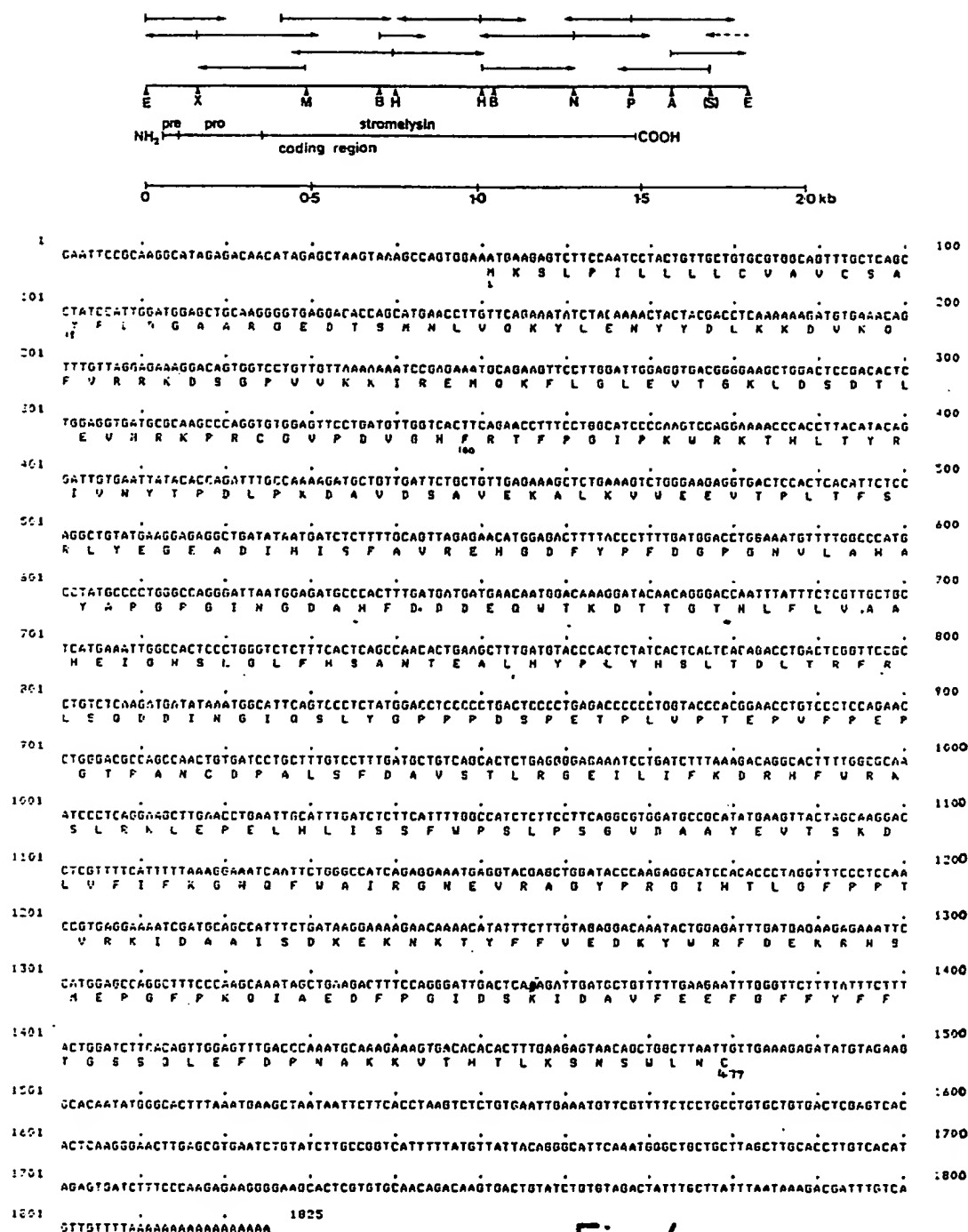
3/7

Fig. 4

Fig. 5

Fig. 9

Fig.6

Counts / min x10⁻³

Time (min)
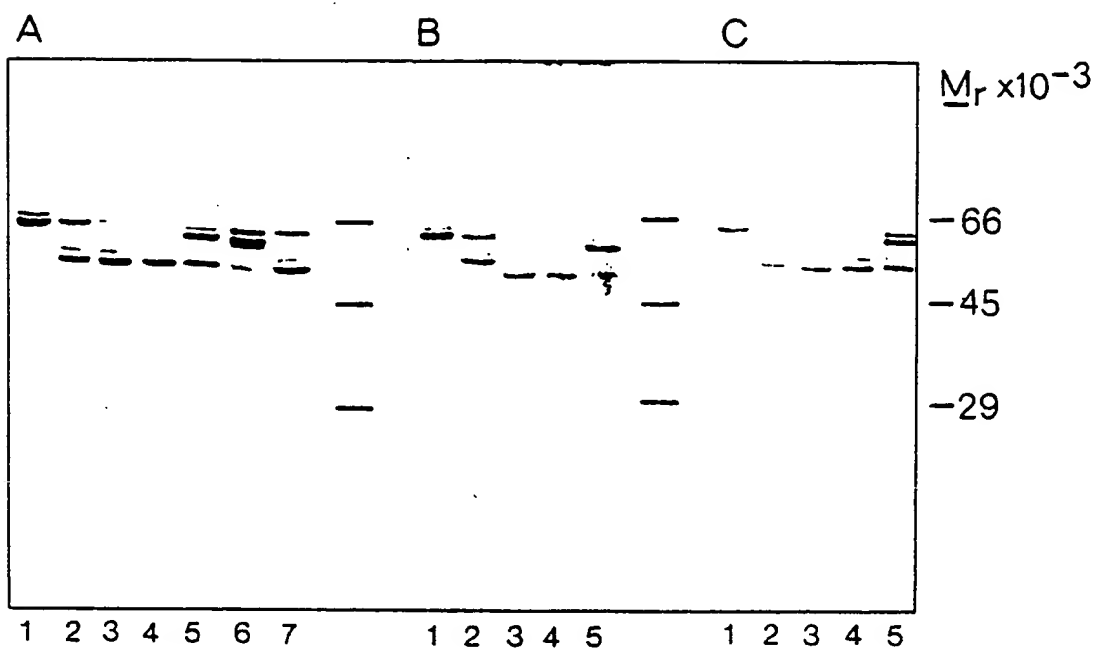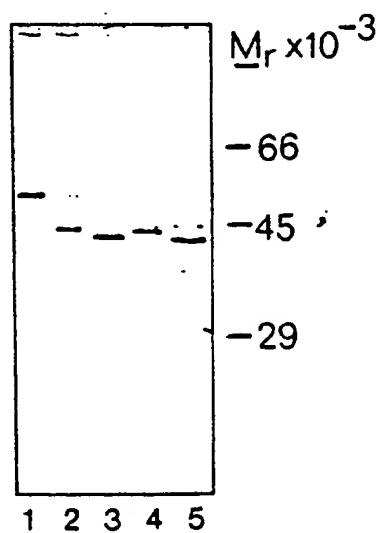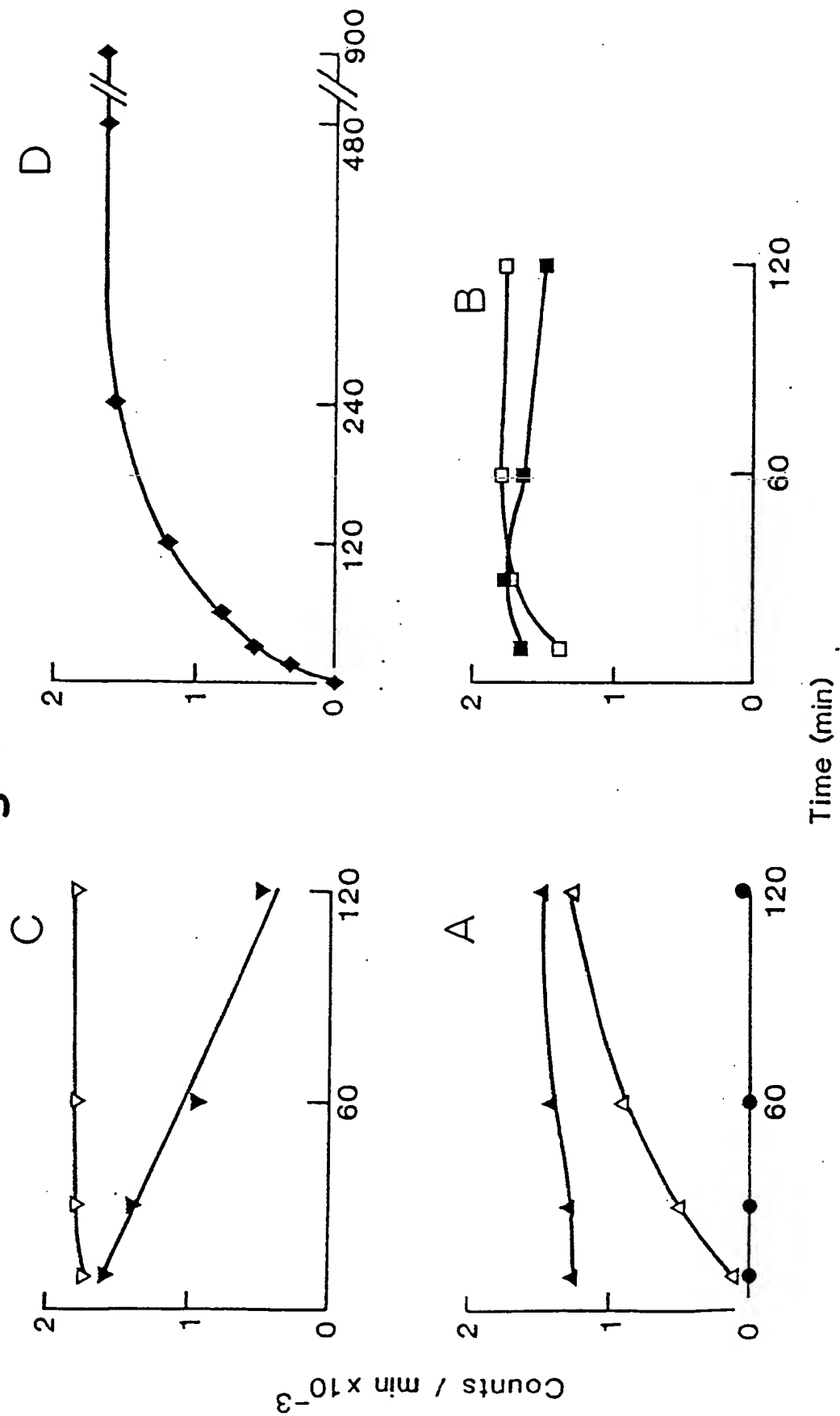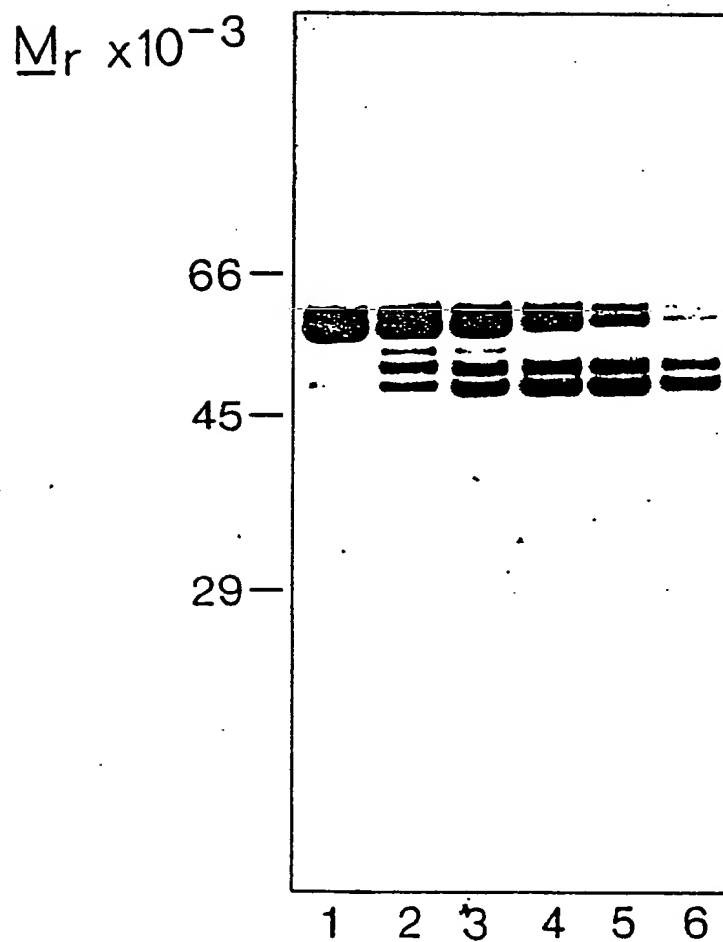
Fig. 7
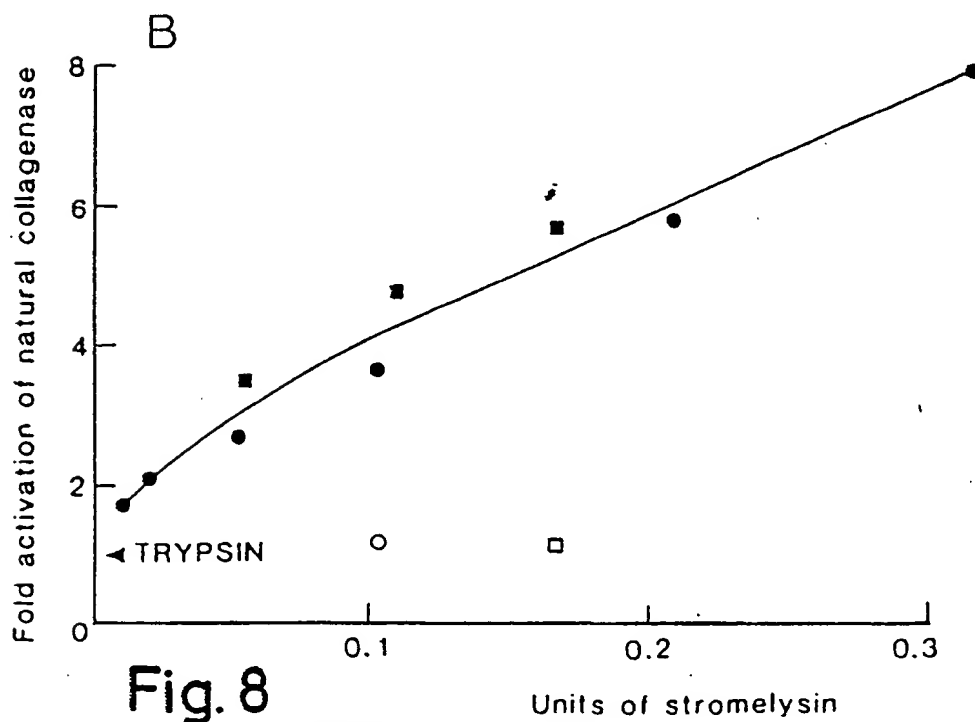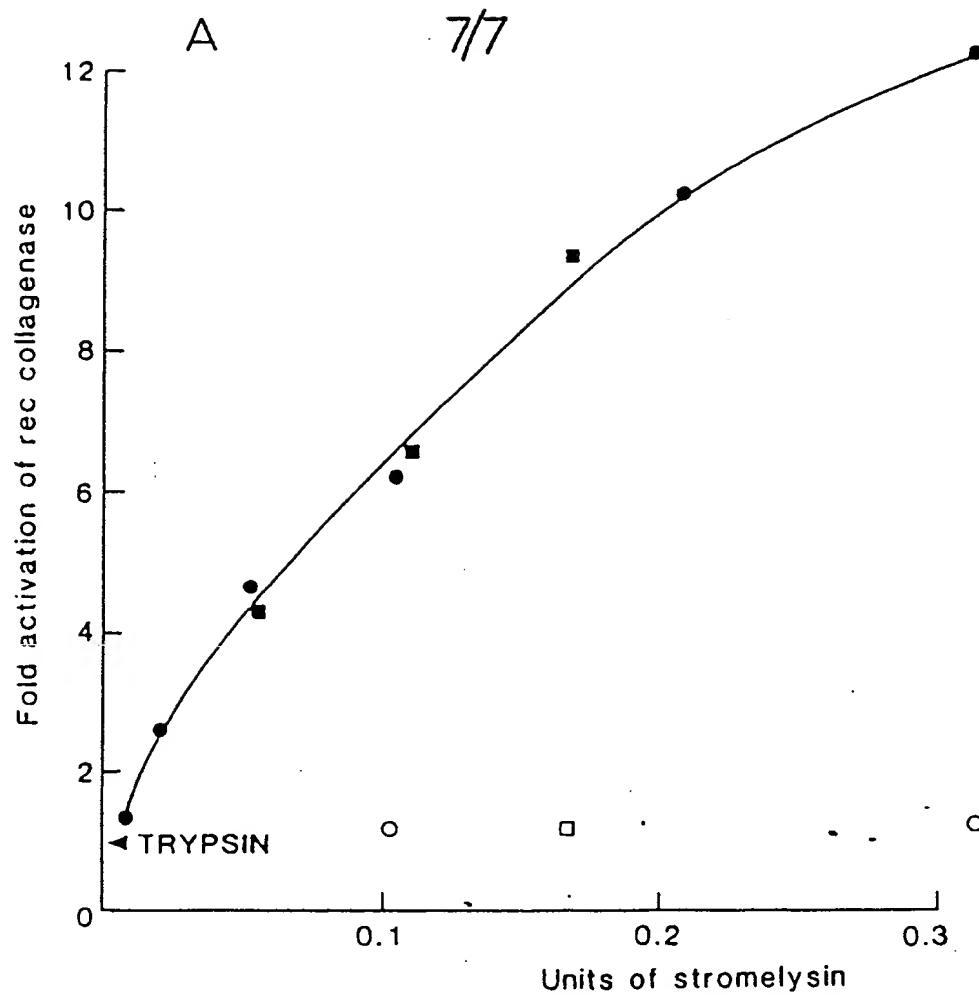
Fig. 8

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 N 15/00; C 12 N 9/64; A 61 K 37/54; C 12 N-1/00

II. FIELDS SEARCHED

Minimum Documentation Searched 7	
Classification System	Classification Symbols
IPC⁴	C 12 N A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	The Journal of Biological Chemistry, volume 260, no. 22, 5 October 1985, The American Society of Biological Chemists, Inc., (US), J.R. Chin et al.: "Stromelysin, a connective tissue-degrading metallo-endopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase", pages 12367-12376 see the whole document	3,4
Y	--	1,2,6-8
X	Biochem. J., volume 209, 1983, The Biochemical Society, (GB), W.A. Galloway et al.: "Purification and characterization of a rabbit bone metalloproteinase that degrades proteoglycan and other connective-tissue components", pages 741-752 see the whole document	3,4
Y	cited in the application	1,2,6-8
	--	./.

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6th October 1987	19 NOV 1987
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The EMBO Journal, volume 4, no. 6, 1985, IRL Press Limited, (Oxford, GB), L.M. Matrisian et al.: "Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts", pages 1435-1440 see the whole document cited in the application --	1,2,6-8
X	J. Cell Biol., volume 97, no. 2, part 5, 1983, S.M. Frisch et al.: "Molecular cloning of a cDNA encoding a secreted connective tissue-degrading metallo-proteinase induced by changes in cytoskeletal structure", page 460a, abstract 1740 see the whole abstract	6
Y	cited in the application --	1,2,7,8
P,X	Biochem. J., volume 240, 1986, (GB), S.E. Whitham et al.: "Comparison of human stromelysin and collagenase by cloning and sequence analysis", pages 913-916 see the whole document --	6
Y	EP, A, 0157604 (SANKYO CO., LTD) 9 October 1985 see the whole document --	1,2,6-8
P,X	Journal of Cell Biology, volume 103, no. 5, part 2, 1986, (US), E.J. Clark et al.: "Synthesis and secretion of collagenase and stromelysin in SV40 transformed human ataxia-telangiectasia fibroblasts", page 257a, abstract 961 see the whole abstract --	6
A	Chemical Abstracts, volume 105, no. 17, 27 October 1986, (Columbus, Ohio, US), S.M. Frisch: "Molecular biology of the stromelysin and collagenase genes", see page 316, abstract 148779t & Diss. Abstr. Int. B 1986, 46(5), 1544 --	
A	Chemical Abstracts, volume 105, no. 3, 21 July 1986, (Columbus, Ohio, US), G.I. Goldberg et al.: "Human fibro-blast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein",../.	

FURTHER INF RMATION CONTINUED FROM THE SECOND SHEET		
	see page 209, abstract 19690h, & J. Biol. Chem. 1986, 261(14), 6600-5	
P,A	GB, A, 2182665 (MONSANTO CO.) 20 May 1987	9,10
A	EP, A, 0115974 (INSTITUT PASTEUR) 15 August 1984 see the whole document	
A	EP, A, 0159497 (K.K. YAKULT HONSHA) 30 October 1985 see the whole document	9,10

V.[X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.[X] Claim numbers ....10.., because they relate to subject matter not required to be searched by this Authority, namely:

See PCT rule 39.1 (IV)   Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2.☐ Claim numbers..........., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out. specifically:

3.☐ Claim numbers..........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/10/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0157604	09/10/85	JP-A- 60207583	19/10/85
GB-A- 2182665	20/05/87	None	
EP-A- 0115974	15/08/84	WO-A- 8402653	19/07/84
		FR-A- 2538702	06/07/84
		AU-A- 2410584	02/08/84
		JP-T- 60500413	28/03/85
		FR-A,B 2538701	06/07/84
EP-A- 0159497	30/10/85	JP-A- 60188066	25/09/85
		US-A- 4624924	25/11/86